Cell Metabolism, Volume *8*

Supplemental Data

T Cells Potentiate PTH-Induced Cortical Bone Loss through CD40L Signaling

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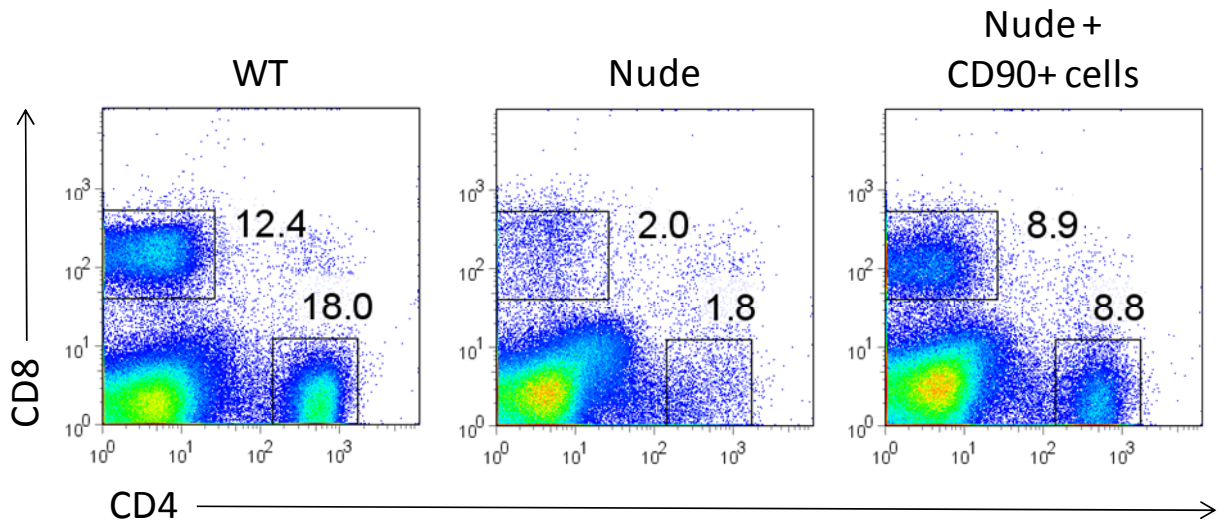


Figure S1. FACS analysis of spleens harvested from WT mice, nude mice, and nude mice subjected to adoptive transfer of WT CD90+ cells two weeks earlier. The adoptive transfer of T cells was carried out by injecting in the tail vein 2 × 10^6 T cells purified by positive immuno-magnetic selection using MACS Microbeads coupled to anti-CD90 (Thy1.2) antibodies. The data show that the adoptive transfer of T cells in nude mice is followed by the homeostatic expansion of donor T cells.

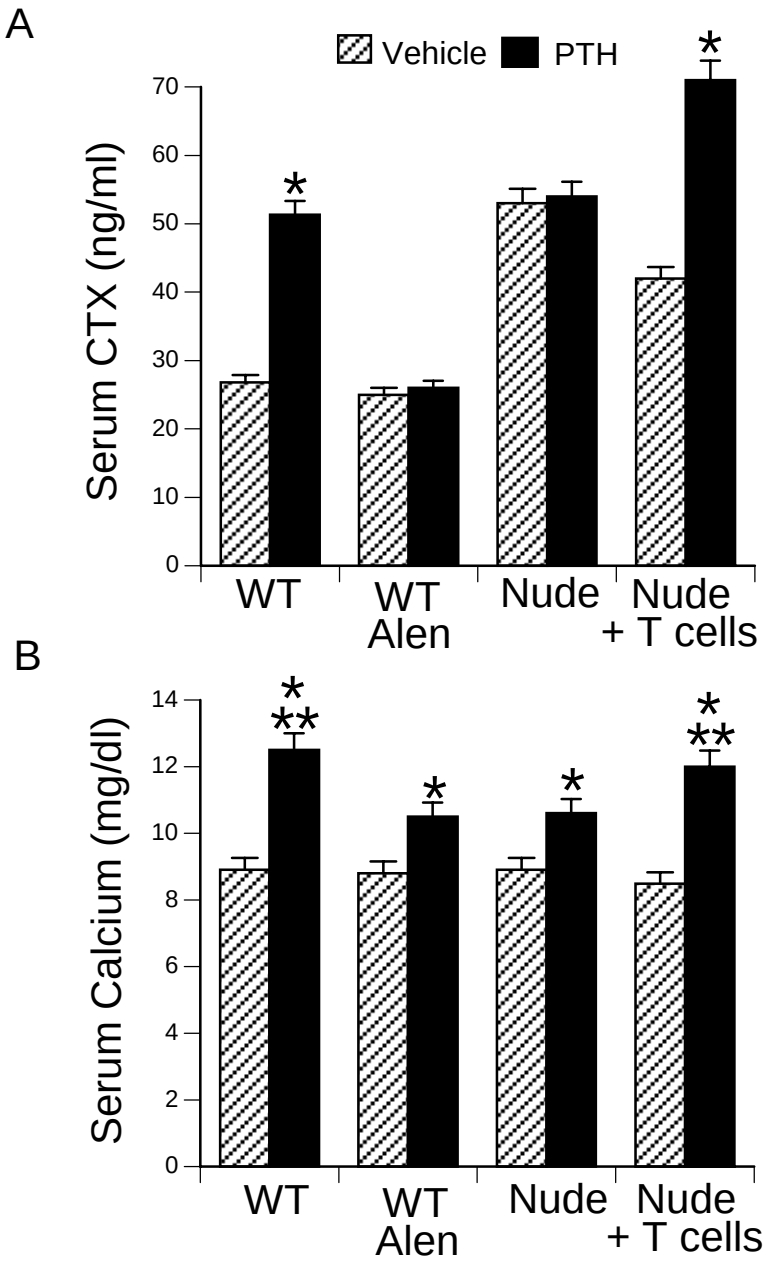

A
Vehicle
PTH
Serum CTX (ng/ml)
70
60
50
40
30
20
10
0
*
*
WT
WT Alen
Nude
Nude + T cells
B
Serum Calcium (mg/dl)
14
12
10
8
6
4
2
0
*
**
*
*
*
**
WT
WT Alen
Nude
Nude + T cells

Figure S2. Effect (mean ± SEM) of cPTH on serum CTX and calcium levels in WT and nude mice. Untreated nude mice and T cell depleted WT mice possess normal serum levels of calcium, indicating that in the absence of T cells endogenous PTH is still capable of exerting calcemic effects. To determine the contribution of skeletal and extra-skeletal mechanisms to the calcemic effects of PTH in T cell replete and T cell deficient mice, WT and nude mice were treated with cPTH at 80 μg/kg/day for 2 weeks. A subset of WT mice was also treated with Alendronate (1.75 mg/kg/wk weekly IP for 4 weeks, starting 2 week before cPTH) to block bone resorption. Additional controls included nude mice previously reconstituted with T cells. Measurements of serum CTX (Panel A) revealed that nude mice have a higher rate of bone resorption. Adoptive transfer of T cells in nude mice was associated with a ~ 20 % decrease in bone resorption. cPTH increased bone resorption in WT controls and T cell reconstituted nude mice, while it failed to do so in T cell deficient nude mice and Alendronate-treated WT mice. Furthermore, PTH caused a small and equal increase in serum calcium in nude mice and Alendronate-treated WT mice (Panel B). The calcemic response observed in these two groups was 2 fold lower than that of control WT mice and T cell reconstituted nude mice. The finding of a blunted, but not abolished, calcemic response in nude mice and Alendronate-treated WT mice suggests that PTH is capable of raising serum calcium through T cell independent extraskeletal effects, such as stimulation of 1,25 $(OH)_2D$ production and renal reclamation of calcium. However stimulation of bone resorption is required for PTH to induce hypercalcemia. * = $p < 0.05$ compared to the corresponding vehicle group. ** = $p < 0.05$ as compared to the other PTH treated groups.

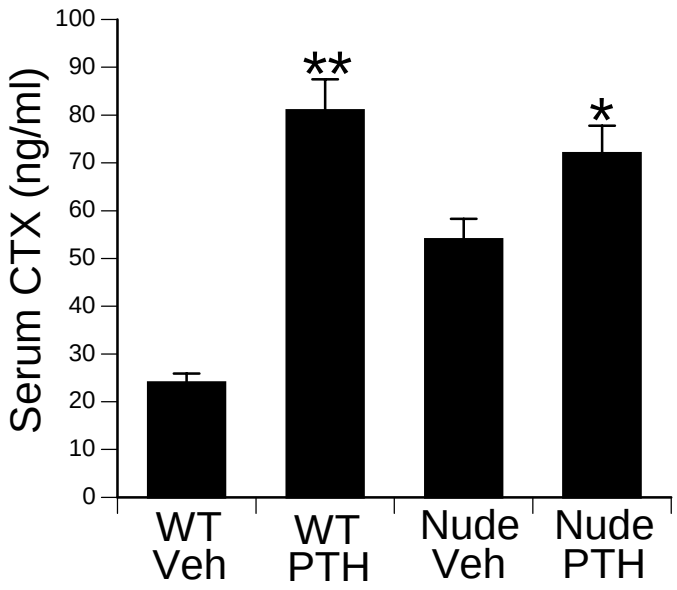


Figure S3. Effect (mean ± SEM) of cPTH at 240 μg/kg/day on serum CTX in WT and nude mice. Transplantation of tumors producing PTH and/or PTHrP in nude mice causes hypercalcemia and increases bone resorption suggesting that the higher dose of circulating PTH/PTHrP attained by transplanting PTH/PTHrP producing tumors as compared to infusing cPTH at 80 μg/kg/day, induces skeletal effects even in the absence of T cells. To test this hypothesis WT and nude mice were infused with cPTH at the doses of 240 and 300 μg/Kg/day. cPTH at 300 μg/kg/day caused the death of all of the WT and nude mice within 5 days. cPTH at 240 μg/kg/day caused the death of 80 % of the WT and 33 % of the nude mice within 7 days due to severe hypercalcemia. Surviving mice had serum PTH levels of 1120 ± 140 ng/L. Infusion of cPTH at 240 μg/kg/day caused a ~330% increase in CTX in WT mice, while it induced a ~30 % CTX rise in nude mice, as compared to the corresponding controls. Thus, while levels of circulating PTH typical of PHP stimulate bone resorption only in the presence of T cells, lethal levels of PTH cause a partial stimulation of bone resorption and hypercalcemia even in the absence of T cells. * = $p < 0.05$ compared to nude vehicle. ** = $p < 0.01$ compared to WT vehicle.

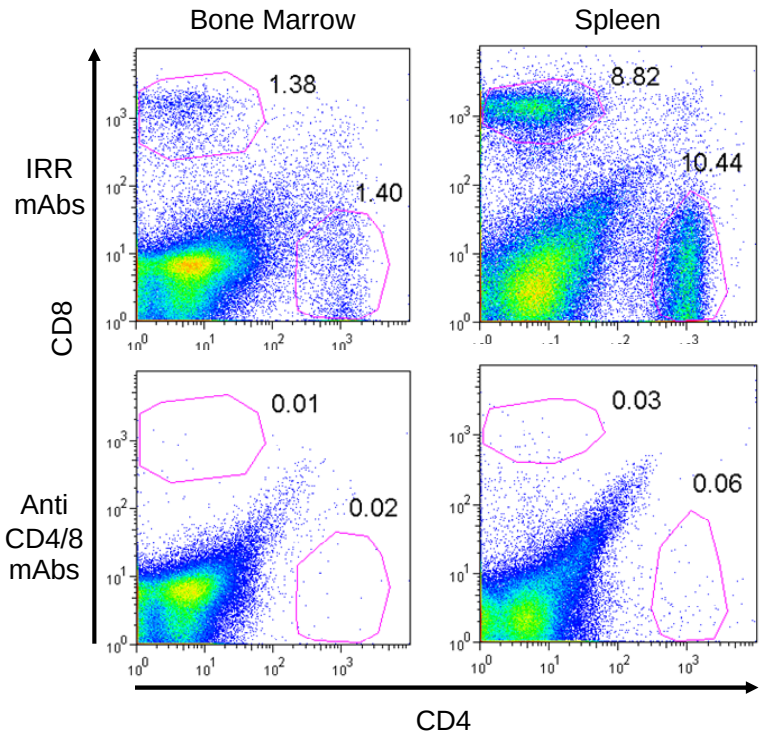


Figure S4. Depletion of bone marrow and spleen T cells by treatment with anti CD4/8 mAbs. WT mice were treated with anti CD4/8 antibodies or isotype matched irrelevant antibodies for 24 days, and continuous PTH (80 μg/kg/day for 2 weeks) or vehicle starting 10 days after the first mAb injection. FACS analysis of bone marrow and spleen samples harvested after 10 days treatment with irrelevant antibodies or anti CD4/8 antibodies. The figure shows CD4+ and CD8+T cells expressed as % of total nucleated cells.

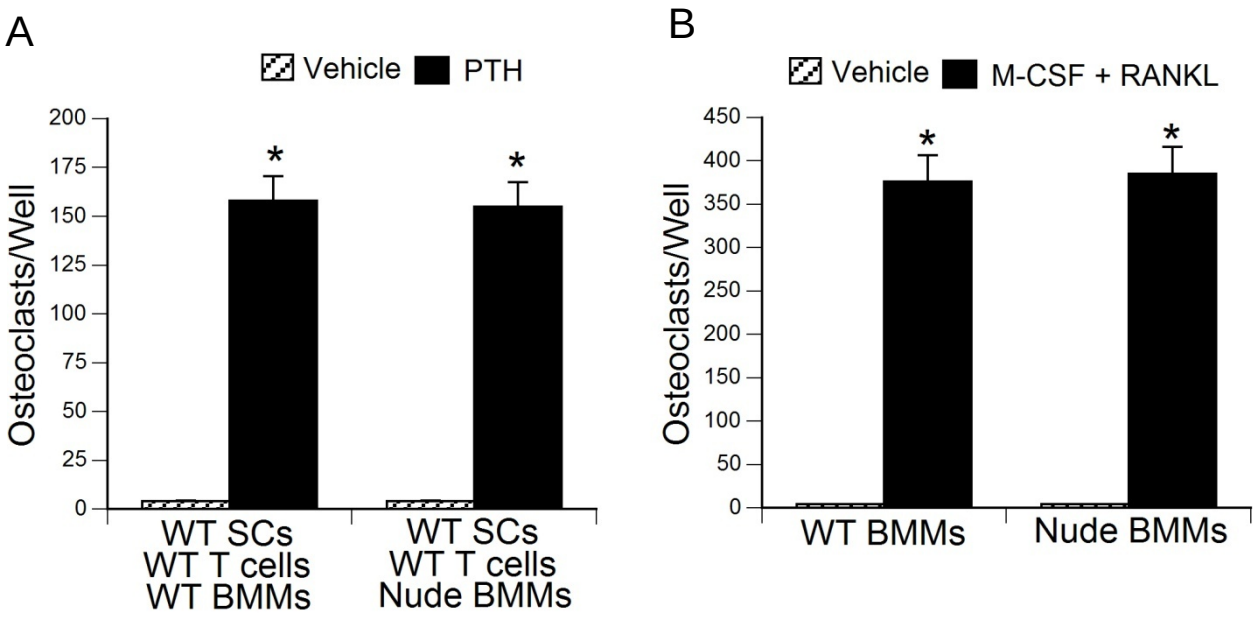
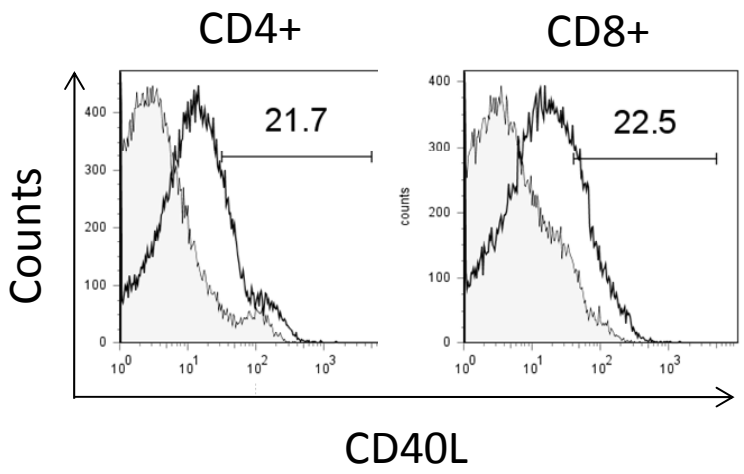
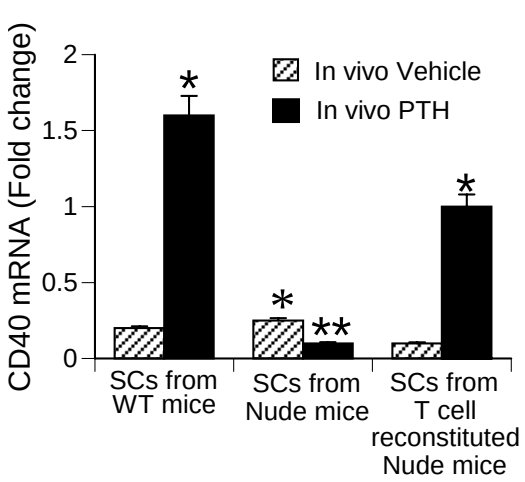


Figure S5. **a** Effect (mean ± SEM) of T cells on BMM osteoclastogenic activity. **A** SCs and T cells from WT mice were cocultured with BMMs from nude and WT mice for 1 week in the presence of PTH (1 nM). Cocultures were stained for TRAP and OCs counted. *= $p<0.05$ compared to vehicle. **B** BMMs from WT and nude mice were cultured for 1 week in the presence of M-CSF (10 ng/ml) and a suboptimal amount of RANKL (25 ng/ml). Cocultures were stained for TRAP and OCs counted. *= $p<0.05$ compared to vehicle

A


CD4+
CD8+
21.7
22.5
Counts
counts
CD40L

B


CD40 mRNA (Fold change)
In vivo Vehicle
In vivo PTH
SCs from WT mice
SCs from Nude mice
SCs from T cell reconstituted Nude mice

C

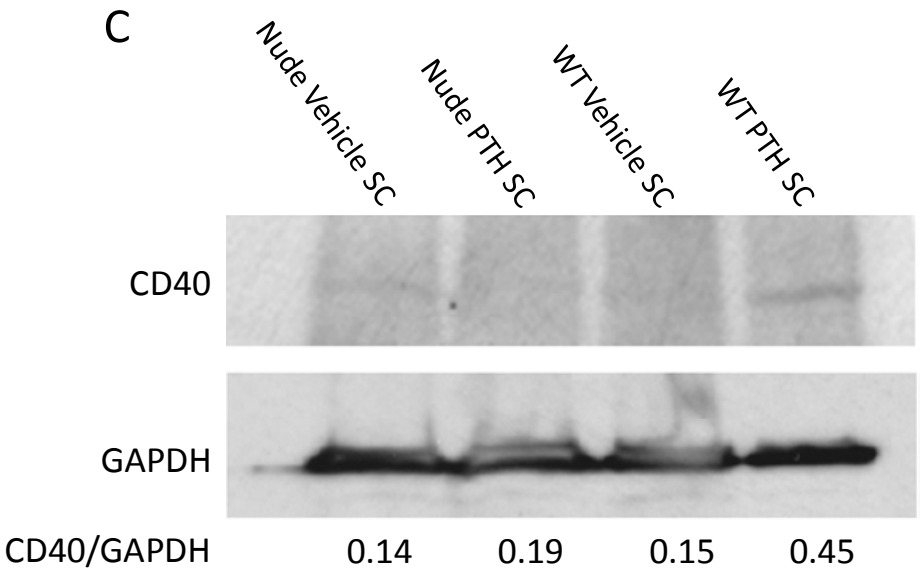

Nude Vehicle SC
Nude PTH SC
WT Vehicle SC
WT PTH SC
CD40
GAPDH
CD40/GAPDH
0.14
0.19
0.15
0.45

Figure S6. **A** Expression of CD40L by CD4+ and CD8+ BM T cells from intact mice. CD4+ and CD8+ T cells were purified by positive immunomagnetic selection, cultured in vitro for 24 hours with plate bound anti-CD3 and anti-CD28 mAbs, and analyzed by FACS. **B** PTH fails to upregulate CD40 mRNA expression in SC from nude mice. WT mice, nude mice, and nude mice previously reconstituted with T cells were treated with vehicle or cPTH for 2 weeks. SCs were then purified, cultured with PTH for 3 days and CD40 expression assessed by real time RT-PCR. Data are expressed as mean $\pm$ SEM *= $p<0.05$ compared to corresponding vehicle group. **= $p<0.05$ compared to T cell replete PTH treated groups. **C** PTH upregulates CD40 mRNA expression in SC from T cell replete BM, but not from T cell depleted BM. WT BM was depleted of T cells in vitro by negative immunomagnetic selection and cultured for 1 week. SCs were then purified, cultured with PTH for 3 days and CD40 expression assessed by real time RT-PCR. Data are expressed as mean $\pm$ SEM. *= $p<0.05$ compared to corresponding vehicle group. **= $p<0.05$ compared to T cell replete PTH treated groups. **D** PTH upregulates CD40 protein levels in SC from WT but not nude mice. WT and nude mice were treated with vehicle or cPTH for 2 weeks. SCs were then purified, cultured with PTH for 3 days and CD40 expression assessed western blotting. Cell membranes were enriched as described (Spina et al., 1988) and protein resolved by SDS PAGE. Following transfer to nitrocellulose membrane, blots were probed with 2 µg/ml rat anti-mouse CD40 antibody (R&D Systems) or rabbit anti-mouse GAPDH and 1:5000 dilution of goat anti-rat or goat anti-rabbit IgG secondary antibody conjugated to HRP (Santa Cruz Biotech.). Protein detection was performed using the ECL chemiluminescence kit (Amersham International). Autoradiographs were scanned on a Flatbed scanner at 600 DPI and densitometry performed using Image J software. Data are expressed as mean $\pm$ SEM *= $p<0.05$ compared to corresponding vehicle group.

Supplemental References

Spina, A., Chiosi, E., Illiano, G., Berlingieri, M.T., Fusco, A., and Grieco, M. (1988). Protein kinase C activities are increased in rat thyroid epithelial cells expressing v-ras genes. Biochem Biophys Res Commun 157, 1093-1103.